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(57) Abstract

Co-amplification and detection of at least three sexually transmitted disease pathogens in DNA isolated from a patient sample are achieved by combining the isolated DNA with at least first, second and third oligonucleotide primer pairs for amplification of a gene fragments from first, second and third different sexually transmitted disease pathogens. One of the primers in each primer pair is labeled with a detectable label. The first, second and third oligonucleotide primer pairs are reacted with the isolated DNA in an amplification reaction to generate an amplification product mixture that contains amplified gene fragments from either or both of the first and second sexually transmitted disease pathogens, depending on whether or not the first and second sexually transmitted disease pathogens are present in the patient sample. Amplified gene fragments in the amplification product mixture are then evaluated, for example by gel electrophoresis to determine whether amplified gene fragments from the first, second or third sexually transmitted disease pathogen are present.

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COMPOSITION, METHOD AND KIT FOR DETECTION AND IDENTIFICATION OF SEXUALLY TRANSMITTED DISEASE MICROORGANISMS

DESCRIPTION

Field of the Invention

This invention relates to combinations of oligonucleotides useful in detecting infectious genitourinary micro-organisms and sexually transmitted disease pathogens such as bacteria, fungi and viruses, by multiplex polymerase chain reaction. The invention also relates to kits containing such combinations and to methods of using said oligonucleotide combination for such detection.

Background of the Invention

Genitourinary tract infections due to infectious microorganisms such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae* are a major cause of morbidity in sexually active individuals. De Schryver, et al. "Epidemiology of sexually transmitted diseases: the global picture" *Bull. W.H.O.* 68:639-654 (1990). In North America, *N. gonorrhoeae* rates are on the increase and *C. trachomatis* is now the most common bacterial sexually transmitted disease (STD) with asymptomatic carriage rates in the general population of 2 to 5% De Schryver et al.; Holmes, et al., *Sexually Transmitted Diseases*, McGraw Hill, New York, pp. 149-166 (19__). Other STDs found in genitourinary samples, such as *Mycoplasma genitalium* and *Ureaplasma urealyticum*, are also etiologic agents of serious genitourinary infection that compromise the health of individuals around the world.

Over the past 15 years, the diagnosis of *C. trachomatis* and *N. gonorrhoeae* infections has been largely dependent on traditional methods such as culture, enzyme immunoassay (EIA) and direct fluorescent antibodies (DFA) for C. trachomatis; and culture, Gram smear and biochemical tests for *N. gonorrhoeae*. Diagnosis of *M. genitalium* and *U. urealyticum* have been generally made by culture.

The last five years have seen major improvements in our ability to detect these STDS with the advent of DNA-hybridization diagnostics and by using the polymerase chain reaction (PCR). PCR is the well known process described in US

Patents 4,683,202, 4,683,195, and 4,965,188, and *Current Protocols in Molecular Biology*, (Eds. Ausubel, F.M. et al, (John Wiley & Sons; 1995)), all of which are incorporated herein by reference, whereby a generally short (50-1000 nt) fragment of a complex DNA sample is reproduced billions of times to generate a substantially pure sample of the fragment DNA. Labeled pathogen specific probes are added to the amplified mixture, and detected if hybridization to the target DNA occurs. Many patents incorporating this technique for hybridization probing and detection of a pathogen have been issued, including US Patents Nos. 5,468,613, 4,683,195, 5,176,995, 5,453,355, 5,217,862 and 5,432,271 and PCT Patent Publications W095/06756, W095/06755, W093/22330, and W090/15159.

Roche Diagnostic Systems, Inc. (Nutley, NJ) manufactures Amplicor™, a test which detects *C. trachomatis* or *N. gonorrohoeae* by the hybridization of a pathogen specific probe to PCR amplified products, detectable by a color change/optical density technique. The test format permits testing a single patient sample for both pathogens using a multiplex PCR amplification, but still requires that two separate detection reactions be performed.

Abbott Laboratories (Abbott Park, IL) makes UriProbe, also a test for *C. trachomatis* and *N. gonorrohoeae*, which relies on the ligase chain reaction (LCR). The LCR method, described in Patent Applications WO 9320227, WO 9300447, WO 9408047, WO 9403636, EP 477 912 uses thermostable ligase enzyme to ligate two DNA probes which hybridize in ligatable juxtaposition on a template DNA strand, thus generating a detectable ligated DNA fragment only if the template DNA is present.

Direct detection of PCR amplified fragments (i.e. without the use of hybridization probes) is not utilized in any of the above noted diagnostic techniques. It would be advantageous to provide a method and compositions that allow the direct detection of PCR amplified fragments, for example, on a high speed automated detection electrophoresis apparatus such as the MicroGene Blaster (Visible Genetics Inc., Toronto).

Further, none of the above-mentioned techniques permit the simultaneous testing for multiple STD's in a single diagnostic reaction. Such a test

would simplify the complex determination of the etiological basis of infection, and would therefore be desirable.

Multiplex PCR (M-PCR) is a technique which permits the amplification of multiple distinct genes or portions of genes in a single reaction mixture. M-PCR is described by Chamberlain et al., "Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification" *Nucleic Acids. Res.* 16: 11141-11156 (1988) and by Edwards et al. "Multiplex PCR" in *PCR primer: A Laboratory Manual* eds. Dieffenbach et al., CSHL Press; Plainview, NY, 1995. M-PCR has been used to distinguish species of *Legionella* (Bej et al. "Multiplex PCR amplification and immobilized capture probes for detection of bacterial pathogens and indicators in water" *Mol. Cell. Probes* 4: 353-365 (1990)), *Mycobacterium* (Wilton et al., "Detection and identification of multiple mycobacterial pathogens by DNA amplification in a single tube" *PCR Methods Appl.* 1: 269-273 (1992)), *Salmonella* (Way et al., "Specific detection of *Salmonella* spp. by multiplex polymerase chain reaction" *Appl. Environ. Microbiol.* 59: 1473-1479 (1993)), *Escherichia coli* and *Shigella* (Bej et al., "Detection of coliform bacteria and *Escherichia coli* by multiplex polymerase chain reaction: Comparison with defined substrated and plating methods for water quality monitoring" *Appl. Environ. Microbiol.* 57: 1473-147 (1991)) and major groups of *Chlamydia* (Kaltenboek et al., "Two-step polymerase chain reactions and restriction endonuclease analyses detect and differentiated ompA DNA of the *Chlamydia* spp." *J. Clin Microbiol.* 30: 1098-1104 (1992)). However, none of these tests provides an M-PCR assay for a plurality of microorganismal STDS. Multiple viruses have been detected by M-PCR, for example in US Pat. No 5,403,107 which is incorporated herein by reference, however, these tests do not provide for direct detection of the amplified fragments of pathogen DNA.

It is an object of the present invention to provide a test methodology which permits a patient sample to be evaluated for the presence of multiple sexually transmitted disease pathogens in a single reaction using M-PCR to simultaneously and in one reaction generate amplification products for all of the subject sexually transmitted disease pathogens present in the sample.

It is a further object of the present invention to provide such a test methodology in which the amplification products are evaluated directly without the use of a secondary hybridization probe.

It is still a further object of the invention to provide specific reagents and kits which can be used in practicing the method of the invention.

Summary of the Invention

The present invention provides a method for co-amplification and detection of at least three sexually transmitted disease pathogens in DNA isolated from a patient sample suspected of containing at least one sexually transmitted disease pathogen. In accordance with the method of the invention, the isolated DNA is combined with at least first, second and third oligonucleotide primer pairs for amplification of a gene fragments from first, second and third different sexually transmitted disease pathogens. One of the primers in each primer pair is labeled with a detectable label. The first, second and third oligonucleotide primer pairs are reacted with the isolated DNA in an amplification reaction to generate an amplification product mixture that contains amplified gene fragments from either or both of the first and second sexually transmitted disease pathogens, depending on whether or not the first and second sexually transmitted disease pathogens are present in the patient sample. Amplified gene fragments in the amplification product mixture are then evaluated, for example by gel electrophoresis, to determine whether amplified gene fragments from the first, second or third sexually transmitted disease pathogen are present.

Detailed Description of the Invention

The instant invention teaches a single test that can simultaneously diagnose the presence of three or more pathogens associated with urethritis and/or sexually transmitted disease (STD) in a patient genitourinary sample. The test detects nucleic acids specific for these pathogens by a modified polymerase chain reaction technique called Multiplex PCR (M-PCR).

PCR Background Information

The PCR technique is quite well known. The following is a brief summary of the diverse information on PCR, and is not meant to detail the information

which is readily available to one skilled in the art, in texts such as *Current Protocols in Molecular Biology*, (Eds. Ausubel, F.M. et al, (John Wiley & Sons; 1995)).

A "primer pair" comprises of a pair of oligonucleotides ("primers") which hybridize to DNA of the target organism. The oligonucleotide is usually synthetically produced. It acts as a point of initiation of DNA synthesis. The exact size and sequence of each primer will vary depending upon the use contemplated, the complexity of the targeted sequence, reaction temperature and the source of the primer. Generally, the primers used in this invention will have from 12 to 60 nucleotides ("nt"), and preferably, they have from 15 to 30 nt.

In conditions suitable for PCR, each primer hybridizes to a different template strand, in an orientation such that the 3' or "extendable" end of each primer points towards each other. The amplification reaction is induced by a polymerization agent (such as a DNA polymerase) and deoxyribonucleotide triphosphates under the appropriate conditions. The result is the formation of primer extension products along the strands, the products having added thereto nucleotides which are complementary to the templates. Once the primer extension products are denatured, one copy of each of the templates has been prepared. By using thermostable enzymes and reagents, cycles of priming, extending and denaturation can be carried out by controlling the temperature of the reaction mixture for as many times as desired to provide an exponential increase in the amount of nucleic acid which has the same sequence as the target nucleic acid. This is the essence of the Polymerase Chain Reaction (PCR). In effect, the target nucleic acid is duplicated (or "amplified") many times so that it is more easily detected.

Although in theory any pair of oligonucleotides which bind to the two strands of duplex DNA in sufficiently close proximity could be used as primers for PCR, there is in practice no guarantee of success in selecting suitable primer sets for PCR. Primers are idiosyncratic and may be influenced by slight changes in salt concentration or temperature. Preferred primers are without internal homology or primer-primer homology. Primers used in the present invention are preferably exactly complementary to the target site of the template DNA. However, it is also possible to employ "substantially complementary" primer sets to obtain amplifications, For example, primers

that form more stable duplexes with the target DNA at the 5'-end of the primer than at the 3'-end thereof can be useful, and can result in less false priming. Mismatched primers tend not to hybridize at higher temperatures. For that reason, it is generally desirable to carry out hybridizations for PCR at the highest possible temperature where both members of the primer set will form stable hybrids with the target site. Expected stability (or melting temperature) of a hybridized primer can be approximated by GC content, since GC base pairs are more stable than AT pairs, or by nearest neighbor thermodynamic parameters. Breslauer et al., "Predictinq DNA duplex stability from base sequence", *Proc. Nat'l Acad. Sci. USA* 83: 3746-3750 (1986).

To evaluate compatibility of primers for use in co-amplification, it is desirable to determine the predicted melting temperature for each primer. This can be accomplished in several ways. For example, the melting temperature, Tm can be calculated using either of the following equations:

Tm(°C) = 81.5 + 16.6 x log [Na] + 0.41 x (%GC)- 675/length where [Na] is the concentration of sodium ions, and the %GC is in number percent, or

$$Tm\ (°C) = 2 X (A + T) + 4 X (G+C)$$

where A, T, G, and C represent the number of adenosine, thymidine, guanosine and cytosine residues in the primer. In general, primers for coaamplification should be selected to have predicted melting temperatures differing by less than 4°C. Notwithstanding the proposed equations for calculating Tm, it is sometimes found that only trial and error can help the investigator determine the actual Tm Of the primer sets selected.

A person skilled in the art knows how to employ the above rules and other rules to design primer sets suitable for amplification of a gene. However, it is widely understood that many theoretically suitable primer sets simply do not work in practice for co-amplifications. In these cases it is necessary to perform an experiment to determine if the amplifications were successful. If amplifications fail, or are non-specific, another primer set needs to be used.

Hybridization sites (or "primer sites") are the sites of hybridization and initiation of synthesis on the target DNA. The sites are selected to be specific to some

part of the target DNA which is unique to the target. The resulting amplified fragment can be any gene fragment, including part of a gene, an entire gene, or contiguous parts of several genes from the target DNA, as long as the hybridization sites of the primer set is specific for that microorganism. It is desirable that the primer sets do not amplify any human DNA which is likely to be contaminating patient samples (mostly patient sample DNA, but also DNA from technicians who handle the sample).

Multiplex PCR Assay for Two or More Disease Organisms

The present invention provides primers sets which permit co-amplification and detection of a plurality of sexually transmitted disease pathogens that may be found in human genitourinary samples in a single test. Such sexually transmitted disease pathogens include but are not limited to *C. trachoma*, *N. gonorrhoeae*, *Mycoplasma genitalium*, *Ureaplasma urealyticum*.

Patient samples suspected of carrying an STD are obtained from a genitourinary body fluid samples such as First Void Urine (FVU), urethral swab, vaginal swab, vaginal introitus swab, forensic sample, or tissue samples (blood or organ, or tumor), Recent studies of nucleic acid amplification technologies such as PCR and LCR have indicated that FVU specimens are the preferred specimen for diagnosing *C. trachomatis* infections in both men and women. FVU is also usually the easiest sample to obtain from patients.

DNA can be isolated from virtually all pathogens found in a FVU sample, by using the technique described in Mahony et al. "Confirmatory polymerase chain reaction testing for *Chlamydia trachomatis* in first void urine from asymptomatic and symptomatic men" *J. Clin. Microbiol.* 30:2241-2245 (1992) which is incorporated herein by reference. The goal of this procedure is to remove unwanted proteins, lipids, carbohydrates and solutes from the sample, leaving only a relatively pure form of DNA taken from the mixture of all micro-organismal and host cells in the sample.

In accordance with the invention, the isolated DNA from a sample is treated with at least two sets of primers to produce amplified gene fragments from different species of sexually transmitted disease pathogens in a multiplex PCR process. Primer sets used in M-PCR are subject to constraints beyond those of PCR alone. For

example, primer sets must successfully co-amplify under the same temperature cycling regime. Primers must not hybridize to other co-amplification products, i.e. they must be specific for single pathogen species only, and should not amplify related or unrelated pathogens. Additional factors applying to the selection of primers for M-PCR are discussed in Rylchik, W., Selection of Primers for Polymerase Chain Reaction", in Methods in Molecular Biology, Vol. 15: PCR Protocols: Current, Methods and Applications, White B.A. ed., Humana press, Totowa, N.J., 1993.

Optimization of reactions can be achieved by modifying reaction variables such as primer concentrations, dNTP amounts, number of thermal cycles, reagent concentrations, sample preparation etc. An optimized result produces approximately the same quantity of reaction product for each fragment when all target organisms are present in the same amounts.

In addition to the above, the length of respective amplification products must be sufficiently different so that respective amplification products can be detected by electrophoretic (size based) separation. For example, the amplification product from a first pathogen is designed to be l00nt; a second pathogen will be 150nt, a third will be 200nt and a fourth will be 250nt. This size difference ensures that when a certain pathogen is not present or is below detectable level, no amplification product is present. The expected fragment length of the amplification product is selected to be specific for each pathogen, and is known from the distance lying between the hybridization sites of the primers in the pathogen target sequence. Differences of only one base in length are sufficient, provided a high resolution gel capable of resolving one base differences is used in analyzing the amplification products. Greater differences in length are preferred.

The primer set selected must also amplify DNA from all strains of the same species. if a primer is situated on a region of variability between strains, some strains will not be detected. A region of DNA that is highly conserved among strains is the preferred primer target sequence. On the other hand, strain specific primer sets could provide useful information if it became clear that certain strains responded to different treatments.

Another primer set representing a control amplification can be included in the reaction mix to monitor the reliability of reaction reagents and sample preparation.

For example, a primer set known to amplify DNA from a commensal microorganism normally found in the type of patient sample being tested (i.e. lactobacillus from vaginal fluid) could be used for a reliable control. Human DNA Primer sets may also be used, since some patient DNA is expected in body fluid samples and tissue samples. An alternative control can be the addition to the patient sample of a known non-human DNA composition, such as a purified plasmid DNA, prior to amplification reactions.

Once the primer sets are determined, they are added to the patient sample DNA in conditions that induce primer extension by PCR (generally requiring the addition of a thermostable polymerase, dNTPs and other reagents). After sufficient thermal cycles, the PCR is halted, and the reaction products, if any, are detected.

Detection of Reaction Products

Detection of the presence of and fragment length of amplification products is facilitated if one or both primers of each primer pair are conjugated to a detectable label. A preferred detectable label is a fluorescent dye, although other labels including radio-labels, chemical couplers such as biotin which can be detected with streptavidin-linked enzymes, and epitope tags such as digoxigenin detected using antibodies available from Boehringer-Mannheim (Mannheim DE) can be used.

Fluorescently-labeled, amplified fragments can be detected using automated fluorescence detection electrophoresis apparatuses such as instruments provided by Visible Genetics Inc. (Toronto, ON), Applied Biosystems Inc. (Foster City, CA), Pharmacia Biotech, Inc. (Piscataway, NJ), Li-Cor, Inc. (Lincoln, NE), and Molecular Dynamics, inc. (Sunnyvale, CA).

A preferred example of a fluorescent label is fluorescein, which is a standard label used in nucleic acid sequencing systems using laser light as a detection system. Further fluorescent labels may include: Rhodamine dyes, cyanine dyes such as CY5 and Cy5.5 (Amersham Life Sciences), dyes with infra-red excitation spectra and many others. These fluorescent labels may be conjugated to the 5' end of the primer using well known methods such as the phosphoramidite (see US Patent No. 4415732, which is incorporated herein by reference) or amino-linker methods.

The use of a different fluorophore attached to the 5' and 3' primers, respectively, can prove advantageous when used in an automated fluorescence detection apparatus with a two-dye detector. The results from one primer can be used as a control to check the results of the other primer. Alternatively, two or more different patient samples could be run in the same channel if one patient's fragments were labeled with one dye, while the other patients' fragments had the different label. This technique would increase the throughput capacity of the apparatus two-fold or more, depending on the number of dyes employed.

Detection of unlabeled amplification products may be made by ethidium bromide staining of such amplification products after electrophoretic separation in an agarose gel. Agarose gel techniques are generally not as precise in their separation as the polyacrylamide gels used in the above noted instruments. However, where amplification products are significantly different in size, such as greater than 40-50 nt, they can often readily be discerned on an agarose gel. Agarose gel electrophoresis and detection techniques are detailed in Protocols in Molec, Biol. at chapter 4, incorporated herein by reference.

Other well known techniques of identifying unlabeled products include Southern blotting, dot blot or slot blot hybridizations. In each of these cases a probe is used to identify the presence and/or location of bands. Such probes may have a radiolabel or chemiluminescent label, and such probes for micro-organismal STDs are known in the art.

Primer Sets Useful with the Invention

The following primer sets have been found to successfully co-amplify DNA fragments from different sexually transmitted disease pathogens present in a patient sample, in the reaction conditions described in the examples below.

Chlamydia trachomatis

Primer set 1

5'-TCC GGA GCG AGT TAC GAA GA-3' (labeled)	SEQ ID NO 1
5'-ATT CAA TGC CCG GGA TTG GT-3'	SEQ ID No 2

Neisseria gonorrhoeae

Primer set 2

5'-GCT ACG CAT ACC CGC GTT GC-3' (labeled)	SEQ ID No 3
5'-CGA AGA CCT TCG AGC AGA CA-3'	SEQ ID NO 4

Mycoplasma genitalium

Primer set 3

5'-AGT TGA TGA AAC CTT AAC CCC TTG G-3' (labeled)	SEQ ID NO 5
5'-CCG TTG AGG GGT TTT CCA TTT TTG C-3'	SEQ ID NO 6

Ureaplasma urealyticum

Primer set 4

5'-GAT CCA ACT TGG ATA GGA CGG-3'	SEQ ID NO 7
5'-GAG ATA ATG ATT ATA TGT CAG GAT CA-3' (labeled)	SEQ ID NO 8

The primers of set I amplify an open reading frame (orf) of the cryptic plasmid of *C. trachomatis*, generating a 241 nt DNA fragment.

The primers of set 2 amplify the cppB gene on the 4.2 kb cryptic plasmid of *N. gonorrhoeae*, generating a 390 nt fragment.

The primers of set 3 amplify part of the MgPa adhesin gene of *M. genitalium*, generating a DNA fragment Of 281 nt.

The primers Of set 4 amplify the urease gene of *U. urealyticum* generating a 167 nt DNA fragment.

The primers used in the present invention are selected to be "substantially complementary" to the specific target nucleic acid priming sites. This means that they must be sufficiently complementary to hybridize with the respective nucleic acid sequences to form the desired hybridization products and then be extendable by a DNA polymerase. In the preferred and most practical situation, the primers have exact complementarity to the nucleic acid sequences of interest.

These primers may be conveniently provided in a kit format. Such a kit may include enough reagents for a single assay or for multiple assays, for example for up to 200 or more assays. The primer sets may be conveniently mixed in a cocktail of primers, with the concentration of each primer optimized for the reaction. Additionally the kit may contain reaction buffer mix, thermostable polymerase such as Taq Polymerase (Perkin-Elmer) or Thermo Sequenase (Amersham Life Sciences), internal standards and/or loading buffer.

The following examples are illustrative of the instant invention and are not meant to be limiting in any way.

EXAMPLE 1

Two hundred FVU specimens (20 ml) were collected from men presenting with non-gonoccocal urethritis to the Nairobi City Commission Special Treatment Clinic. Two urethral swabs and a FVU specimen were collected for culture, EIA and PCR as described previously. The FVU specimen was collected first. The study was approved by the University of Nairobi's Scientific and Ethical Review Committee.

DNA was prepared from the FVU specimens as described previously in Mahony, et al., *J. Clin. Microbiol.* 30: 2241-2245 (1992).

The optimal conditions for M-PCR were established by varying the concentrations of $MgCl_2$, dNTPs, and primers at different annealing temperatures. The optimal conditions determined were an annealing temperature of 55°C, primer concentrations of 0.5 uM, a $MgCl_2$ concentration of 2.5 uM and dNTP concentrations of 200 uM. After the addition of Taq polymerase (Amersham Life Sciences), the reaction mixtures were thermal cycled as follows: 94°C for two minutes then 40 cycles of 60 sec at 55°C; 120 sec at 72°C; and 60 sec at 94°C. The final extension phase was allowed to proceed for 300 sec before being placed on ice at 4°C.

Primers used for the detection of *C. trachomatis* were:

Primer set 1

5'-TCC GGA GCG AGT TAC GAA GA-3' SEQ ID NO 1

5'-ATT CAA TGC CCG GGA TTG GT-3' SEQ ID NO 2

which amplify a 241 bp fragment of the genetically conserved plasmid. These primers are also referred to as KL1-KL2. Mahony, et al., *J. Clin. Microbiol.* 30:2241-2245 (1992).

Primers used for the detection of *N. gonorrhoeae* were:

Primer set 2

5'-GCT ACG CAT ACC CGC GTT GC-3' SEQ ID NO 3

5'-CGA AGA CCT TCG AGC AGA CA-3' SEQ ID NO 4

which amplify a 390 bp fragment of the cppB gene on the 4.2 Kb cryptic plasmid. These primers are also referred to as HO1-H03. Mahony, et al., *J. Clin, Microbiol.* 30:2241-2245 (1992).

PCR products were analyzed by agarose gel electrophoresis and Southern blot hybridization using fluorescein-11-dUTP labeled oligonucleotide probes prepared with terminal deoxynucieotidyl transferase using an ECL 3'-oligolabeling and detection kit (Amersham Life Sciences, Arlington Heights, Ill). The hybridization probes used were the KL3 probe specific for *C. trachomatis* 241 bp PCR product and the JMGC3 probe derived from the published plasmid sequence, Korch, et al., "Cryptic plasmid of Neisseria *gonorrhoeae*: Complete nucleotide sequence and genetic organization", *J. Bacteriol.* 163: 430-438 (1985), and specific for the 390 bp NG-PCR product.

Extensive precautions were employed for all PCR assays to prevent carryover contamination. These included the use of i) designated biosafety containment hoods for preparing specimens, setting up PCRS, and a separate area for analyzing products; ii) plugged pipette tips or positive displacement pipettors; iii) several negative controls interspersed with clinical specimens; iv) periodic swabbing of work areas to detect amplified DNA; and v) the judicious use of a confirmatory PCR in resolving discordant results.

Under these optimal conditions the total analytical sensitivity of M-PCR was 10 fg of total DNA equivalent to 1-2 genome copies for both *C. trachomatis* and *N. gonorrhoeae* and equal to that of the individual CT-PCR and NG-PCR assays. The

bands observed in the gel demonstrated the specificity of the primers used in M-PCR; KL1-KL2 primers amplified only *C. trachomatis* DNA while HOI-HO3 primers amplified only *N. gonorrhoeae* DNA in the presence of both sets of primers and DNA from both organisms.

The ability of M-PCR to detect CT and NG simultaneously in urine specimens with high sensitivity provides a new non-invasive approach to the diagnosis of urethritis in men.

EXAMPLE 2

This protocol detects four bacteria associated with urethretis and/or sexually transmitted diseases (STDs): *Chlamydia trachomatis*, *Mycoplasma genitalium*, *Neisseria gonorrhoeae*, and *Ureaplasma urealyticum* in a single M-PCR reaction.

FVU samples are prepared for PCR amplification as follows. Deposit 100 ul Of FVU in a sterile microcentrifuge tube. Centrifuge at 12,000 x g for 20 min and remove the supernatant. Add 100 ul of Lysis Solution (Proteinase K @ 100 ug/ml; 1% Tween 20) to the bacteria pellet. Incubate sample for 1 h at 55°C, or 18 h at room temperature. Incubate further at 95°C for 10 min. Add 200 ul of Geneclean II glass milk. Elute the DNA in 10 ul of ddH_20. A lysis solution control can be made as follows: add the lysis solution to a sterile tube (a tube without any urine pellet), and treat this tube like the others.

PCR amplification is obtained for 10 reactions (including 10% extra for the loss during pipetting) as follows: Take a sterile 1.5 or 2.0 ml tube. Add 88 ul of the PCR Master mix (per 880 ul: add 275 ul of 10x PCR buffer without $MgCl_2$ (Perkin-Elmer); 275 ul of $MgCl_2$ @ 25 mM (Perkin-Elmer); 330 ul of each dNTPs @ 2.5 mM). Add 6.9 ul of the Primer Mix in which each of the following primers are present at 2 um: The following primers are present:

5'-TCC GGA GCG AGT TAC GAA GA-3' (labeled)	[SEQ ID No. 1]
5'-ATT CAA TGC CCG GGA TTG GT-3'	[SEQ ID No. 2]
5'-GCT ACG CAT ACC CGC GTT GC-3' (labeled)	[SEQ ID No. 3]
5'-CGA AGA CCT TCG AGC AGA CA-31	[SEQ ID No. 4]

5'-AGT TCA TGA AAC CTT AAC CCC TTG G-3'(labeled) [SEQ ID No. 5]

5'-CCG TTG AGG GGT TTT CCA TTT TTG C-31 [SEQ ID No. 6]

5'-GAT CCA ACT TGG ATA GGA CGG-3' [SEQ ID No. 7]

5'-GAG ATA ATG ATT ATA TGT CAG GAT CA-3'(labeled) [SEQ ID No. 8]

The label for use with a Visible Genetics Inc. MicroGene Blaster, is a Cy5.5 fluorophore (Amersham Life Sciences) amino-linked to the 5' end nucleotide.

Add 151.2 ul of sterile ddH_20. Prepare PCR reaction tubes (0.2 Ul tubes, thin walls), label and set them in the rack. (If not using a heated lid thermocycler, add one drop of mineral oil to each PCR tube.) When ready to put the reaction mix into the PCR tubes, add 1.4 ul of Taq DNA polymerase @ 5 U/ul. Mix well and use mix as soon as possible. Put 22.5 ul of the solution prepared above in each PCR tube. Add 2.5 ul of sample to each tube. Include the following controls: One negative control per 5 samples. This negative control contains 2.5 ul of water instead of 2.5 ul of sample. One lysis buffer control: add 2.5 ul of the lysis solution to make sure that the lysis solution was not contaminated. Close each tube as soon as the sample has been added. Alternate negative controls with samples: one negative control per 5 samples. When all the samples have been added, put the tube in the thermal cycler. Thermal cycles proceed as follows:

94°C/3 min then 35 cycles of the following:

94°C/30 sec

60°C/30 sec

72°C/45 sec

after these cycles, then continue with

72°C/5 min

4°C/until ready to load.

Add 5 ul of the Internal Standard mix (described below) to each PCR tube and pipet two or three times to mix. Take 5 ul and add it to 5 ul of the MicroGene Blaster Loading Buffer. Store the rest of the PCR reaction at –20°C or 4°C. Immediately

before loading, heat the sample/loading buffer to 75°C for 3 min. Cool rapidly on ice and load 2 ul on a single lane of the MicroGene Blaster.

The size of the various peaks in a run are as follows:

- 138-bp (internal standard)
- 167-bp (U. urealyticum)
- 200-bp (internal Standard)
- 241-bp (C. trachomatis)
- 281-bp (M. genitalium)
- 351-bp (internal standard)
- 390-bp (N. gonorrhoeae)
- 426-bp (internal standard)

Internal Standards

The Internal Standards are provided in a solution containing 4 fragments, labeled with CY5-5, that is added to the PCR tube after amplification. 4 fragments of appropriate length can be made from the plasmid template pGEM-7Zf(-) using the following primers:

Forward Primer

T7 (2983-4; Tm.- 58°C; labeled):

5'-GTA ATA CGA CTC ACT ATA GGG C-3' [SEQ. ID No. 9]

Reverse primers

GEM99 (120-99; Tm: 65°C):

5'-TAC TCA AGC TAT GCA TCC AAC G-3' [SEQ. ID No. 10]

GEMI6I(182-161; Tm = 61°C):

5'-ATT TCA CAC AGC AAA CAG CTA T-3' [SEQ. ID No. 11]

GEM312 (333-312; Tm= 70°C):

5'-ATT AAT GCA GCT GGC ACG ACA G-3' [SEQ. ID No. 12]

GEM387 (408-387; Tm= 74°C):

5'-GCA GCG AGT CAG TGA GCG AGG A-3' [SEQ. ID No. 13]

The following annealing temperature can be used:

T7-GEM99 (generates a 138-bp fragment): 52.5°C

T7-GEM161 (generates a 200-bp fragment): 52.2°C

T7-GEM312 (generates a 351-bp fragment): 53.3°C

T7-GEM387 (generates a 426-bp fragment): 54.3°C

Each fragment can be generated separately, purified (by gel or otherwise), and calibrated to give good peaks on the MicroGene Blaster. These peaks can be used to calibrate both length and quantity of fragments observed.

SEQUENCE LISTING

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(iii) NUMBER OF SEQUENCES: 13

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette - 3.5 inch, 1.44 Mb storage

(B) COMPUTER: IBM compatible

(C) OPERATING SYSTEM: MS DOS

(D) SOFTWARE: Word Perfect

(vi) CURRENT APPLICATION DATA

(A) APPLICATION NUMBER:

(B) FILING DATE:

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(A) APPLICATION NUMBER:

(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

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(B) REGISTRATION NUMBER: 32,038

(C) REFERENCE/DOCKET NUMBER: VGEN.P-031-US

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(C) TELEX:

(2) INFORMATION FOR SEQ ID NO: 1;

(I) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: C. trachomatis

(D) OTHER INFORMATION: primer for C. trachomatis

(xi) SEQUENCE DESCRIPTION. SEQ ID NO:I:

TCCGGAGCGA GTTACGAAGA 20

(2) INFORMATION FOR SEQ ID NO: 2:

(I) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: C. trachomatis

(D) OTHER INFORMATION: primer for C. trachomatis

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATTCAATGCC CGGGATTGGT 20

(2) INFORMATION FOR SEQ ID NO: 3:

(I) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Neisseria gonorrohoeae

(D) OTHER INFORMATION: primer for Neisseria gonorrhoeae

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCTACGCATA CCCGCGTTGC 20

(2) INFORMATION FOR SEQ ID NO: 4:

(I) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE: gonorrhoeae

(A) ORGANISM: Neisseria gonorrhoeae

(D) OTHER INFORMATION: Primer for Neisseria gonorrhoeae

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGAAGACCTT CGAGCAGACA 20

(2) INFORMATION FOR SEQ ID NO: 5:

(I) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Mycoplasma genitalium

(D) OTHER INFORMATION: primer for Mycoplasma genitalium

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGTTGATGAA ACCTTAACCC CTTGG 25

(2) INFORMATION FOR SEQ ID NO: 6:

(I) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Mycoplasma genitalium

(D) OTHER INFORMATION: primer for Mycoplasma genitalium

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCGTTGAGGG GTTTTCCATT TTTGC 25

(2) INFORMATION FOR SEQ ID NO: 7:

(I) SEQUENCE CHARACTERISTICS;

(A) LENGTH: 21

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Ureaplasma urealyticum

(D) OTHER INFORMATION: Primer for Ureaplasma urealyticum

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATCCAACTT GGATAGGACG G 21

(2) INFORMATION FOR SEQ ID NO: 8:

(I) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Ureaplasma urealyticum

(D) OTHER INFORMATION: primer for Ureaplasma urealyticum

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
GAGATAATGA TTATATGTCA GGATCA 26

(2) INFORMATION FOR SEQ ID NO: 9:
(I) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: no
(iv) ANTI-SENSE: yes
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:
(A) ORGANISM:
(D) OTHER INFORMATION: primer for pGEM-7Zf(-)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
GTAATACGAC TCACTATAGG GC 22

(2) INFORMATION FOR SEQ ID NO: 10:
(I) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: no
(iv) ANTI-SENSE: no
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:
(A) ORGANISM:

(D) OTHER INFORMATION: Primer for pGEM-7Zf(-)

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TACTCAAGCT ATGCATCCAA CG 22

(2) INFORMATION FOR SEQ ID NO: 11:

(I) SEQUENCE CHARACTERISTICS:

(A) LENGTH, 22

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: No

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(D) OTHER INFORMATION: primer for pGEM-7Zf(-)

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

ATTTCACACA GGAAACAGCT AT 22

(2) INFORMATION FOR SEQ ID NO: 12:

(I) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22

(E) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(V) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(D) OTHER INFORMATION: primer for pGEM-7Zf(-)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:
ATTAATGCAG CTGGCACGAC AG 22

(2) INFORMATION FOR SEQ ID NO: 13:
(I) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: No
(v) FRAGMENT TYPE, internal
(vi) ORIGINAL SOURCE:
(A) ORGANISM:
(D) OTHER INFORMATION: primer for pGEM-7Zf(-)
(xi) SEQUENCE DESCRIPTION SEQ ID NO: 13:
GCAGCGAGTC AGTGAGCGAG GA 22

CLAIMS

1. A method for specifically detecting the presence of sexually transmitted disease pathogens which may be present in a sample comprising the steps of:

a) isolating DNA from a patient sample suspected of containing at least one sexually transmitted disease pathogen;

b) adding to a single aliquot of the isolated DNA first, second and third oligonucleotide primer pairs, each of said primer pairs being effective to amplify a diagnostic portion of a gene from first, second and third different sexually transmitted disease pathogen selected from the group consisting Of *Chlamydia trachomatis, Mycoplasma genitalium, Neisseria gonorrhoeae,* and *Ureaplasma urealyticum* wherein at least one primer in each primer pair is labeled with a fluorescent label;

c) reacting the oligonucleotide primer pairs with the isolated DNA in an amplification reaction to generate an amplification product mixture, said amplification product mixture containing amplified gene fragments from the first, second and third sexually transmitted disease pathogens, when said first, second and third sexually transmitted disease pathogens are present in the patient sample; and

d) evaluating any amplified gene fragments in the amplification product mixture by detection of the fluorescent label, wherein the presence of an amplified gene fragment from one or more of the sexually transmitted disease pathogens identifies the presence of that sexually transmitted disease pathogen in the patient sample.

2. The method of claim 1, wherein the fluorophore is Cy5.5.

3. The method of claim 1, wherein the oligonucleotide primers in first and second primer pairs have a melting temperature within 10 C of each other.

4. The method of claim 1, wherein said first and second oligonucleotide primer pairs are selected from the group consisting of:

1)	5'-TCC GGA GCG AGT TAC GAA GA-3'	SEQ ID NO 1
	5'-ATT CAA TGC CCG GGA TTG GT-3'	SEQ ID NO 2

b) 5'-GCT ACG CAT ACC CGC GTT GC-3' SEQ ID NO 3

5'-CGA AGA CCT TCG AGC AGA CA-3' SEQ ID NO 4

c) 5'-AGT TGA TGA AAC CTT AAC CCC TTG G-3' SEQ ID NO 5

5'-CCG TTG AGG GGT TTT CCA TTT TTG C-3' SEQ ID NO 6

and d) 5'-GAT CCA ACT TGG ATA GGA CGG-31 SEQ ID NO 7

5'-GAG ATA ATG ATT ATA TGT CAG GAT CA-3' SEQ ID NO 8.

5. The method according to claim 1, wherein a fourth oligonucleotide primer pair for amplification of a gene fragment from a fourth sexually transmitted disease pathogen different from the first, second and third sexually transmitted disease pathogens is added to the isolated DNA and reacted with the isolated DNA at the same time as the first, second and third oligonucleotide primer pairs.

6. The method of claim 5, wherein said first, second, third and fourth oligonucleotide primer pairs are:

2) 5'-TCC GGA GCG AGT TAC GAA GA-3' SEQ ID NO 1

5'-ATT CAA TGC CCG GGA TTG GT-3' SEQ ID NO 2

b) 5'-GCT ACG CAT ACC CGC GTT GC-3' SEQ ID NO 3

5'-CGA AGA CCT TCG AGC AGA CA-3' SEQ ID NO 4

c) 5'-AGT TGA TGA AAC CTT AAC CCC TTG G-3' SEQ ID NO 5

5'-CCG TTG AGG GGT TTT CCA TTT TTG C-3' SEQ ID NO 6

and d) 5'-GAT CCA ACT TGG ATA GGA CGG-31 SEQ ID NO 7

5'-GAG ATA ATG ATT ATA TGT CAG GAT CA-3' SEQ ID NO 8.

7. A kit for the simultaneous detection of at least three sexually transmitted disease micro-organisms comprising first, second and third oligonucleotide primer pairs, each of said primer pairs being effective to amplify a gene fragment from a different sexually transmitted disease pathogen selected from the group consisting of Chlamydia trachomatis, mycoplasma genitalium, Neisseria gonorrhoeae, and Ureaplasma urealyticum, wherein at least one primer of each oligonucleotide primer pair is labeled

with a flourescent label, and wherein the gene fragment produced by amplification of DNA using each primer pair has a different length from that produced by amplification of DNA using the other primer pairs.

8. The kit of claim 7, wherein said fluorescent label is Cy5.5.

9. The kit of claim 7, wherein the oligonucleotide primers in first and second primer pairs have a melting temperature within 10 C of each other.

10. The kit of claim 7, wherein the first, second and third oligonucleotide primer pairs are selected from the group consisting of:

3)	5'-TCC GGA GCG AGT TAC GAA GA-3'	SEQ ID NO 1
	5'-ATT CAA TGC CCG GGA TTG GT-3'	SEQ ID NO 2
b)	5'-GCT ACG CAT ACC CGC GTT GC-3'	SEQ ID NO 3
	5'-CGA AGA CCT TCG AGC AGA CA-3'	SEQ ID NO 4
c)	5'-AGT TGA TGA AAC CTT AAC CCC TTG G-3'	SEQ ID NO 5
	5'-CCG TTG AGG GGT TTT CCA TTT TTG C-3'	SEQ ID NO 6
and d)	5'-GAT CCA ACT TGG ATA GGA CGG-31	SEQ ID NO 7
	5'-GAG ATA ATG ATT ATA TGT CAG GAT CA-3'	SEQ ID NO 8.

11. The kit according to claim 7, further comprising a fourth oligonucleotide primer pair for amplification of a gene fragment from a fourth sexually transmitted disease microorganism different from the first, second and third sexually transmitted disease microorganisms.

12. The kit of claim 11, wherein said first, second, third and fourth oligonucleotide primer pairs are:

4)	5'-TCC GGA GCG AGT TAC GAA GA-3'	SEQ ID NO 1
	5'-ATT CAA TGC CCG GGA TTG GT-3'	SEQ ID NO 2
b)	5'-GCT ACG CAT ACC CGC GTT GC-3'	SEQ ID NO 3
	5'-CGA AGA CCT TCG AGC AGA CA-3'	SEQ ID NO 4

c) 5'-AGT TGA TGA AAC CTT AAC CCC TTG G-3' SEQ ID NO 5

5'-CCG TTG AGG GGT TTT CCA TTT TTG C-3' SEQ ID NO 6

and d) 5'-GAT CCA ACT TGG ATA GGA CGG-31 SEQ ID NO 7

5'-GAG ATA ATG ATT ATA TGT CAG GAT CA-3' SEQ ID NO 8.

13. A composition for detection of sexually transmitted diseases comprising at least three primer pairs selected from the group consisting of

5) 5'-TCC GGA GCG AGT TAC GAA GA-3' SEQ ID NO 1

5'-ATT CAA TGC CCG GGA TTG GT-3' SEQ ID NO 2

b) 5'-GCT ACG CAT ACC CGC GTT GC-3' SEQ ID NO 3

5'-CGA AGA CCT TCG AGC AGA CA-3' SEQ ID NO 4

c) 5'-AGT TGA TGA AAC CTT AAC CCC TTG G-3' SEQ ID NO 5

5'-CCG TTG AGG GGT TTT CCA TTT TTG C-3' SEQ ID NO 6

and d) 5'-GAT CCA ACT TGG ATA GGA CGG-31 SEQ ID NO 7

5'-GAG ATA ATG ATT ATA TGT CAG GAT CA-3' SEQ ID NO 8.

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(57) Abstract

Co-amplification and detection of at least three sexually transmitted disease pathogens in DNA isolated from a patient sample are achieved by combining the isolated DNA with at least first, second and third oligonucleotide primer pairs for amplification of a gene fragments from first, second and third different sexually transmitted disease pathogens. One of the primers in each primer pair is labeled with a detectable label. The first, second and third oligonucleotide primer pairs are reacted with the isolated DNA in an amplification reaction to generate an amplification product mixture that contains amplified gene fragments from either or both of the first and second sexually transmitted disease pathogens, depending on whether or not the first and second sexually transmitted disease pathogens are present in the patient sample. Amplified gene fragments in the amplification product mixture are then evaluated, for example by gel electrophoresis to determine whether amplified gene fragments from the first, second or third sexually transmitted disease pathogen are present.

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DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/CA 97/00660

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category ³	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MAHONY J ET AL: "Detection of C. trachomatis (CT), N. gonorrhoeae (NG), U. urealyticum (UU), M. genitalium (MG), in urine specimens by multiplex PCR (M-PCR)" ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 96, no. 0, 19 - 23 May 1996, page 9 XP002056565 see whole abstract C-47. --- -/--	1-11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

³ Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 February 1998	10/03/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 125, no. 19, 4 November 1996 Columbus, Ohio, US; abstract no. 239517k, ZENG, XINGWU ET AL: "Rapid detection of mixed infection of Neisseria gonorrhoeae, Chlymidia trachomatis, Ureaplasma urealyticum by multiplex-PCR " page 271; column left; XP002056582 see abstract & ZENG, XINGWU ET AL: ZHONGHUA YIXUE JIANYAN ZAZHI, vol. 19, no. 3, February 1996, pages 176-78, ---	1-11
Y	MITRANI-ROSENBAUM S ET AL: "SIMULTANEOUS DETECTION OF THREE COMMON SEXUALLY TRANSMITTED AGENTS BY POLYMERASE CHAIN REACTION" AMERICAN JOURNAL OF OBSTETRICS & GYNECOLOGY, vol. 171, no. 3, September 1994, pages 784-790, XP000612760 see the whole document ---	1-3
Y	DE BARBEYRAC B ET AL: "Microbiological study of pelvic inflammatory disease (PID): Report on a french cohort" ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 96, no. 0, 19 - 23 May 1996, page 286 XP002056566 see abstract G-26 ---	1-3
Y	STEWART R ET AL: "Use of digoxigenin dUTP to increase detection sensitivity of PCR assays for common sexual diseases" ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 94, no. 0, 23 May 1994 - 27 May 1995, page 568 XP002056567 see abstract C-438 ---	1-3
Y	EP 0 687 737 A (HOFFMANN LA ROCHE) 20 December 1995 see claim 13 ---	1-3
A	EP 0 648 845 A (EASTMAN KODAK CO) 19 April 1995 -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/CA 97/00660

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0687737 A	20-12-95	US 5508168 A	16-04-96
		CA 2149257 A	17-11-95
		JP 2692702 B	17-12-97
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		US 5674717 A	07-10-97